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Biomimetic Networks For Selective Recognition of Biomolecules

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ABSTRACT

Studies of protein binding domains reveal molecular architectures with specific chemical moieties that provide a framework for selective recognition of target biomolecules in aqueous environment. By matching functionality and positioning of chemical residues, we have been successful in designing biomimetic polymer networks that specifically bind biomolecules in aqueous environments. Our work addresses the preparation, behavior, and dynamics of the three-dimensional structure of biomimetic polymers for selective recognition via non-covalent complexation. In particular, the synthesis and characterization of recognitive gels for the macromolecular recognition of D-glucose is highlighted. Novel copolymer networks containing poly(ethylene glycol) (PEG) and functional monomers such as acrylic acid, methacrylic acid, and acrylamide were synthesized in dimethyl sulfoxide (polar, aprotic solvent) via UV-free radical polymerization. Polymers were characterized by single and competitive equilibrium and kinetic binding studies, single and competitive fluorescent and confocal microscopy studies, dynamic network swelling studies, DPC, and FE-SEM. Results qualitatively and quantitatively demonstrate effective glucose-binding polymers in aqueous solvent. Due to the presence of template, the template mediated polymerization process resulted in a more macroporous structure as exhibited by dynamic swelling experiments, confocal microscopy, and SEM. Recognitive networks had a more macroporous structure with absorption of water occurring via non-fickian diffusion at a faster rate and with a higher equilibrium value. Polymerization kinetic studies suggest that the template molecule has more than a dilution effect on the polymerization, and the effect of the template is related strongly to the rate of propagation. The processes and analytical techniques presented are applicable to other biologically significant molecules and recognitive networks, in which hydrogen bonding, hydrophobic, or ionic contributions will direct recognition. Further developments are expected to have direct impact on applications such as analyte controlled and modulated drug and protein delivery, drug and biological elimination, drug targeting, tissue engineering, and micro- or nano-devices.

INTRODUCTION

At this stage in the evolving field of biomaterials science, major effort is being directed toward engineering the architectural design of biomaterials on a molecular level. By controlling recognition and specificity, the preparation of *synthetic macromolecular gels with designed artificial recognitive domains* is soon to be the next hurdle crossed in polymer and biomaterials development. Therefore, the next generation of biomaterials will include recognitive oriented design and intelligent complexation mechanisms that originate from specific macromolecular chemistry, itself present in the polymer matrix or surface in a controlled manner, which

manipulate the surface and bulk properties of the material in reproducible and tunable ways. The interest in this technology stems from a larger interest within our laboratory in artificially duplicating complex biological and physiological processes such as biomolecule modulated drug and protein release [1], targeted drug and protein delivery (site or ligand-specific interaction with cells and tissues), biomolecule recognition and removal of undesirable biologicals [2], ligand-directed cooperative allosteric recognition processes, directed on-off ligand binding processes, tissue engineering devices, biosensors, and novel therapeutic and micro-sensing devices, etc. For example, surface patterning of recognitive gels on silicon substrates can create micro- or nano-binding regions with areas of differing chemistry [3], which would be the basis for micro- or nano-diagnostic, drug delivery, or tissue engineering devices [4].

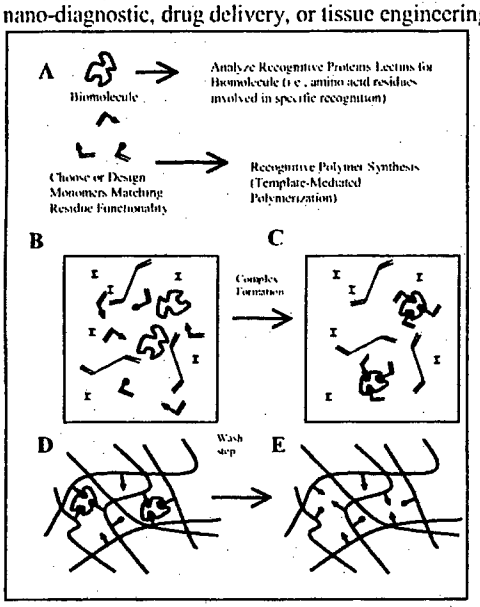


Figure 1: Biomimetic Approach to Producing Recognitive Networks

A: Mimic recognitive proteins and enzymes by analyzing the amino acids involved in binding a particular molecule and duplicating complexation interactions. **B:** Solution mixture of biomolecule (template), functional monomer(s) (triangles and circles), crosslinking monomer, solvent, and initiator (I). **C:** The pre-polymerization complex is formed via covalent or non-covalent chemistry. **D:** The formation of the network (imprinting process). **E:** Wash step where original template is removed.

Our goal, in general, is to produce stereo-specific, three-dimensional binding cavities for biologically significant molecules that function in aqueous environments. By tailoring the polymer network architecture and composition, effective biomimetic recognition sites can be created in polymer gels (Figure 1).

The network structure (polymer morphology, porosity, diffusional characteristics) depends upon the type of monomer chemistry (anionic, cationic, neutral, amphiphilic), the association interactions between monomers and pendent groups, the solvent, and the relative amounts of comonomers in the feed from which the structure is formed. Recognitive success, i.e., the ability to correlate high template binding affinity and specificity, depends on the relative amount of cross interaction between the solvent and the intended interactions. For non-covalent complexation, this translates to the strength of hydrogen bonding, hydrophobic interactions, π-π orbital interactions, ionic interactions, and van der Waals forces employed during template-monomer complex formation.

If proper complexation occurs in the pre-polymerization stage, the network formation will proceed with effective recognitive domains. For example, many binding proteins contain non-covalent binding mechanisms (e.g., hydrogen bonding, hydrophobic interactions) that bind specific molecules quite well in water

(polar, protic solvent) [5]. Our scientific rationale is based on the hypothesis that effectively designed recognitive networks will have superior binding properties and directed recognition in aqueous environments by properly tuning the non-covalent pre-polymerization complexation interactions between the gel functionality and template biomolecule (increasing or decreasing macromolecular chain hydrophobicity [6], including strong hydrogen bond donors and acceptors [7], or including strong ionic directed recognition sites [8]).

EXPERIMENTAL

Materials

Methacrylic acid (MAA), acrylic acid (AA), acrylamide (Aam), dimethylsulfoxide (DMSO), and D-glucose were purchased from Aldrich (Milwaukee, WI). Poly(ethylene glycol) 200 dimethacrylate (PEG200DMA) was obtained from Polysciences, Inc. (Warrington, PA). Irgacure® 184, 1-hydroxycyclohexyl phenyl ketone, was purchased from Ciba Specialty Chemicals (Tarrytown, NY). Fluorescent D-glucose analogue, 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxy-glucose (2-NBDG), was purchased from Molecular Probes, Inc.

Methods: D-Glucose Recognitive Network Synthesis

In a typical experiment involving Aam or AA as functional monomer (monomers were selected to match corresponding glucose binding protein residues of aspartate, glutamate, and asparagine), D-glucose, Aam or AA, and 2.0 mL of DMSO were mixed together. Then PEG200DMA and 1.5 wt% Irgacure® 184 were added to the mixture. Feed monomer compositions of this type varied from 20 to 80 mole% Aam per mole monomers (or 50 to 85 mole% AA) as well as from 4 to 15 mole % D-glucose. Control polymers were made with exactly the same composition except D-glucose was not added.

After preparation, the solution was placed in a nitrogen atmosphere and nitrogen was bubbled for 30 minutes. Polymerizations occurred between glass microscope slides (75 x 50 x 1mm, Fisher Scientific, Pittsburgh, PA) using 0.5 mm, 0.7mm, or 0.035 mm Teflon® spacers in a nitrogen atmosphere at a UV intensity of 10.0-15.0 mW/cm^2 for 15 minutes (UltraCure 100, EFOS Inc., Ontario, Canada). Polymers were placed in deionized water for 24 hours, then carefully separated from the slides, and were cut into various diameter discs using a cork borer. Discs were then placed in 50 mL conical tubes and placed on a rotating mixer (25 RPM, 70 degree angle, Glas-Col, Terre Haute, IN) and resuspended within multiple 24 hour wash steps (2 washes in acetic acid/deionized water (1:5 ratio); 8 washes in deionized water) to remove template and excess monomer. The resulting discs were then dried in air at ambient conditions and placed in a vacuum oven (T=26 °C, 28 mm Hg vacuum) until a constant weight was obtained (less than 0.1 wt% difference). The discs were then stored in a dessicator until testing.

Methods: Recognition, Selectivity, and Dynamic Swelling Studies

In a typical binding study, a known amount of the template solution (e.g., D-glucose and D-galactose in competitive binding studies) was added to an aqueous solution containing a known amount of polymer discs. Samples were placed on a rotating mixer (70 degree angle; 25 RPM)

and supernatant was sampled at equilibrium. The time for equilibrium to occur in each system was predetermined by separate kinetic binding studies, where supernatant samples were taken at different time points. Equilibrium and kinetic-binding results were quantitatively calculated by HPLC measurements of the resulting supernatant (Phenomenex RPM monosaccharide column (300 x 7.8 mm), DI water mobile phase, 0.6 ml/min flow rate, temperature 80°C, Shimadzu RID-10A refractive index detector). Competitive binding results were visualized using a fluorescent glucose analogue, 2-NBDG (Figure 2). The analogue was added to vials containing a known amount of polymer (maximum absorption 466 nm; maximum emission 542 nm). A Nikon Labophot fluorescent microscope with a FITC filter set was used and images were acquired with an Optronics 470T CCD camera and captured using MetaMorph software from Universal Imaging. By analyzing a large amount of pixels (N=10,000) within these images, a histogram of intensity values was obtained (Adobe Photoshop). Confocal analysis was performed using a Bio-Rad MRC 1024 Confocal Microscope with an MRC 1024 system. Images, z-sections, etc. were collected using LaserSharp software and image analysis was conducted using Confocal Assistant software. The equilibrium swelling behavior of the imprinted gels was studied by weighing dry samples and placing them in a known volume of solution with and without template. The gels were weighed by removing the gels at specific periods of time and blotting with filter paper to remove excess surface solvent.

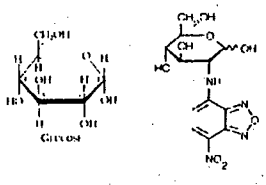


Figure 2: D-Glucose - Template and Fluorescent Analogue. Competitive binding results were visualized using a fluorescent glucose analogue 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) -2-deoxy-glucose (2-NBDG, Molecular Probes, Inc.). In solution, glucose mutarotates between two conformations: alpha (as shown, 30-35%) and beta (65-70%) position of the carbon 1 hydroxyl group at equilibrium. D-Galactose only differs by the carbon 4 hydroxyl position.

RESULTS AND DISCUSSION

We have been successful in synthesizing novel glucose-binding gels based on non-covalent interactions (hydrogen bonding, hydrophobic interactions) formed within polar, aprotic solvent (DMSO).

For AA and Aam functional networks, a bound ratio (amount D-glucose bound recognitive network/control network) greater than one indicates that glucose was memorized within the gel compared to a randomly polymerized network (bound ratios in water were 3.4 and 5.0 for Aam-PEG200DMA and AA-PEG200DMA networks, respectively). It is important to note that the control polymer will bind some amount of template (i.e., will contain some randomly introduced, properly positioned functional groups). For AA and Aam networks, the choice of DMSO as solvent during polymerization increased the bound ratio compared to aqueous solvents during polymerization (DMSO is aprotic and does not have the ability to be a hydrogen bond donor and interfere with complex formation).

Figure 3 provides a kinetic aqueous binding analysis of glucose-imprinted Aam-PEG200DMA copolymers prepared in DMSO. The recognitive to non-imprinted (control) bound ratio is 3.4, demonstrating the memorization of glucose within the network. Also, the equilibrium binding data was well represented by a linear isotherm at low concentration ranges (i.e., <1 mg/ml D-glucose).

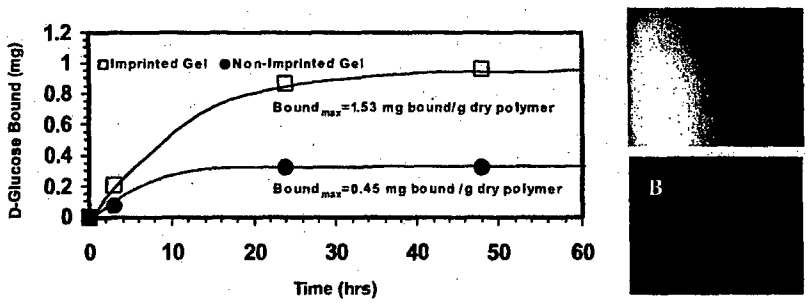


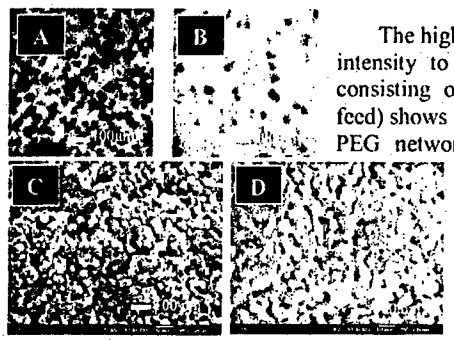
Figure 3: Kinetic D-Glucose Binding Study in Water. Acrylamide-PEG200DMA Copolymers of 67% Crosslinking Ratio Prepared in DMSO (T=24°C). **Fluorescent D-Glucose Analogue Binding in Water.** Acrylic Acid–PEG200DMA Copolymers with 67% Crosslinking Ratio Prepared in DMSO. **A:** Recognitive Polymer (I=208.84+/-6.48 (10,000 pixels)). **B:** Control Polymer (I=36.28+/-1.26 (10,000 pixels)).

Typically, the difference between recognitive and non-imprinted polymer binding values increased as the cross-linking ratio (mole crosslinking monomer/mole total monomers) increased. These results suggest that the use of PEGDMA instead of EGDMA (the average number of ethylene glycol units is 4.5 and 1 for PEG200DMA and EGDMA, respectively) can be beneficial to the diffusional character of the imprinted networks and retain memorized recognition toward template molecules.

For improved analysis of fluorescent intensities, thin polymer discs (diameter, 5.5 mm; thickness, 35μm) were prepared. By analyzing fluorescent intensity values from polymer discs of equal thickness, a histogram of intensity values was obtained, which provided quantitative analysis of binding (Table I). This data contains fluorescent profiles from competitive binding analysis of Aam-PEG200DMA copolymers with varying amounts of D-glucose added to a given amount of fluorescent analogue (FITC filter cube, 1/8 integration time, 4x objective). These results were analyzed carefully by matching as many parameters as possible during analysis (excitation time, objective and field of view, camera integration time, etc.). The results demonstrate the effectiveness of fluorescent tracing in relation to a competitive analogue molecule. As glucose concentration is increased and fluorescent analogue is held at constant concentration, the fluorescent intensity of the gel decreases. Thus, glucose competed and filled binding sites once occupied by the fluorescent analogue.

Table I. Competitive D-Glucose Substrate Binding in Water: Acrylamide-PEG200DMA Copolymers with 67% Crosslinking Ratio (prepared in DMSO).

Competitive Substrate	Recognitive Intensity	Control Intensity
Fluorescent Analogue Only (Concentration = F_g)	223 +/- 11.81	50.97 +/- 0.77
F. Analogue and Glucose (100 x F_g)	99.44 +/- 4.36	80.77 +/- 3.95
F. Analogue and Glucose (3,000 x F_g)	49.81 +/- 1.09	48.85 +/- 0.88
F. Analogue and Galactose (100 x F_g)	220 +/- 10.10	na
F. Analogue and Galactose (3,000 x F_g)	209.06 +/- 10.46	na
Fluorescent Analogue Only (PEG only network)	na	56.57 +/- 0.90



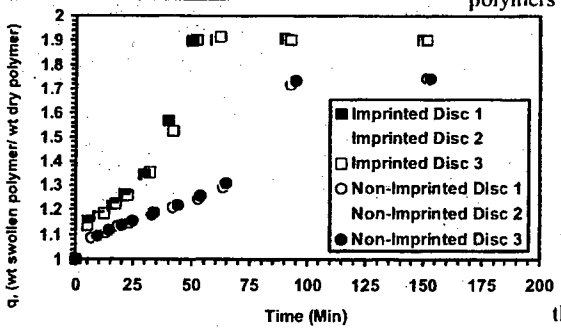


Figure 4: Dynamic Network Swelling Study in Water and Polymer Morphology via Confocal Microscopy Transmission /FE-SEM. Acrylamide-PEG200DMA Copolymers with 67 % Crosslinking Ratio Prepared in DMSO.**A.** Recognitive Network. **B.** Control Network. **C.** Recognitive FE-SEM **D.** Control FE-SEM

The highest glucose ratio ($3{,}000xF_g$) drops the fluorescent intensity to level consistent with control polymer. A gel consisting of PEG200DMA only (i.e., no functionality in feed) shows that there is a low level of interaction between the PEG network and the fluorescent analogue. Competitive binding studies between D-glucose and 2-NBDG yielded conclusive results that D-glucose is bound to this polymer system and not the 2-NBDG fluorophore. AA functionalized networks exhibited similar behavior (Figure 3 A, B).

The selectivity of the Aam-PEG200DMA polymers was determined by varying concentrations of D-galactose added to a constant concentration of D-glucose fluorescent analogue (Table I). The results suggest polymers selective to D-glucose since the fluorescent intensity remained approximately unchanged.

Figure 4 shows dynamic swelling data (without template present) from Aam-PEG200DMA copolymers. The imprinted gels swelled at a faster rate than the non-imprinted gels displaying non-Fickian swelling behavior (i.e., anomalous transport since swelling process is not dominated by the polymer viscoelastic relaxation time or water diffusion) and exhibiting a higher degree of equilibrium swelling. As both systems adsorbed water, the discs became increasingly opaque, with the non-imprinted gels displaying a higher degree of translucence. The water swelling tests correlate the properties of the network (homogeneity and porosity) to the imprinting process. Due to the presence of template, the imprinting process resulted in a more porous structure as exhibited by these studies and confocal microscopy/FE-SEM (Figure 4 A, B, C, D).

The formation of a three-dimensional network is a kinetically controlled process that depends on the functionality, reactivity, and concentration of the monomeric components. Traditionally, crosslinking monomers with similar reactivities to the chain building monomer are selected to produce a homogeneous network with spatially even crosslinking density. Differences in the reactivity of the monomers can lead to structural heterogeneity. With imprinting, the template functional monomer complex influences the polymerization conditions and the resulting polymer network. The influence of the complex formation on the resulting network has not been explored and is currently being studied by our group. Polymerization kinetic studies suggest that

the template molecule has more than a dilution effect on the polymerization, and the effect of the template is related strongly to the rate of propagation.

CONCLUSIONS

The studies presented in this paper aid in further understanding and optimizing template-mediated polymerization processes for the production of novel recognitive networks for biologically significant molecules. Based on a biomimetic approach, we have synthesized novel imprinted gel structures for biomolecule recognition. In particular, we have been successful in producing and characterizing recognitive networks for the recognition of D-glucose. Equally important in this endeavor is the potential to produce recognitive networks for a wide range of biomolecules providing hydrogen bonding, hydrophobic, or ionic contributions direct recognition. Thus, these techniques and the resulting polymers can be tailored to recognize a broad range of molecules in aqueous environment. Currently, using similar techniques, our group is producing and optimizing recognitive networks for a variety of drugs and biological molecules, including proteins.

Developments of particular interest are expected to be wide and far reaching and cover a broad range of materials, such as intelligent biomolecule-modulated drug and protein delivery, nano-scale patterning and recognition of biological molecules for diagnostic and therapeutic devices, site or ligand-specific interaction with cells and tissues for targeting applications and tissue engineering, and biosensors.

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